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Journal of Chromatography A, 1098 (2005) 177–182
JOURNAL OF CHROMATOGRAPHY A
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Separation and determination of tetrandrine and fangchinoline in herbal medicines by flow injection-micellar electrokinetic capillary chromatography with internal standard method

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Received 5 June 2005; received in revised form 29 July 2005; accepted 23 August 2005
Available online 12 September 2005

Abstract

A simple, rapid and precision flow injection-micellar electrokinetic capillary chromatography (FI-MEKC) system with trimethoprim as internal standard (IS) for automated quantitative analysis of tetrandrine (TET) and fangchinoline (FAN) in various herbal medicines was demonstrated. The real sample throughput was 19–40 samples per hour using the background electrolyte (BGE) containing 15 mM acetic acid–15 mM sodium acetate–3% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20)–5% (v/v) methanol at pH 5.5. The method resulted in excellent linearity, with correlation coefficient of regression equation of 0.9996 and 0.9991 for TET and FAN, respectively. Recoveries were in the range 95–109% and 92–106% for TET and FAN, respectively.

Keywords: Flow injection-micellar electrokinetic capillary chromatography; Internal standard method; Tetrandrine; Fangchinoline; Herbal medicines

1. Introduction

Tetrandrine (TET) and fangchinoline (FAN) are two alkaloids that exist in Radix *Stephaniae tetrandrae* S. Moore. They have long been known to show many pharmacological activities including anti-allergic, inhibiting the release of histamine, promoting phagocytosis, dilating coronary artery and decreasing myocardial oxygen consumption, inhibiting the growth of *Bacillus tuberculosis*, *Staphylococcus aureus* and *ameba*, and anti-cancer activities. The analytes are important due to their toxicity in humans and livestock as constitutes of poisonous plants at higher concentrations, and the two alkaloids exhibit potentially useful pharmacological activities at lower concentrations [1]. So, it is necessary to establish a suitable analytical method to evaluate or control the quality of these herbal preparations.

Several methods such as high-performance liquid chromatography (HPLC) [2,3], thin-layer chromatography (TLC) [4], capillary electrophoresis (CE) [5] and non-aqueous CE [6] have been reported for the determination of TET and FAN. However, TLC lacks quantitative precision, while HPLC presents lower efficient and time-consuming. Although CE has the advantages of high resolution capability and small sample volume, the discontinuous sample introduction mode confined the sample throughput and precision. The combined FI-CE system has the favorable potentials in achieving efficient continuous sample introduction [7–15], including enhanced sampling frequencies, improved reproducibility. Additionally, the microchip with an H-channel design in this work presented a low-cost alternative for more basic studies on a microfluidic system [16–21].

However, in FI-CE, sample was introduced into the separation capillary by electrokinetic means. The quantity of solutes injected depends on their electrophoretic mobilities; so, a sampling bias exists with electrokinetic injection [22], and the peak areas plotted versus analyte concentrations often show poor linearity, making the quantitative determination imprecise. Therefore, the quantitation by help of an IS and the measurement of the peak ratio between analyte and IS is always recommended. The aim of this work studies the applicability of FI-MEKC system with IS for determination TET and FAN in herbal medicines. Because trimethoprim has similar behavior as the analytes in the electric field and its occurrence in the herbal medicines is not likely, it was used as an IS in this paper.

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doi:10.1016/j.chroma.2005.08.067

2. Experimental

2.1. Chemicals and materials

TET, FAN and trimethoprim (IS) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products, China. For their chemical structures, see Fig. 1. The standards were used as received. The crude drug of *S. tetrandra* S. Moore was purchased from local drug stores. Feng Tong An capsule were purchased from Tonghua Jinhui Medicine Corporation (Tonghua, Jilin, China). Methanol was purchased from Tianjin Secondary Chemical Factory (Tianjin, China). Acetic acid was purchased from Tianjin Chemical Corporation (Tianjin, China). Sodium acetate was purchased from Beijing Chemical Plant (Beijing, China). Tween 20 was purchased from Beijing Chemical Company (Beijing, China). All chemicals were of analytical reagent grade and were used as received. All solution and buffer were made in distilled water.

2.2. Solutions preparation

Stock standard solutions (400 μg/mL) of TET and FAN were prepared in 50% aqueous ethanol. The standard solutions at various concentrations were prepared by appropriate dilution of the stock solution with 50% aqueous ethanol, which contained trimethoprim spiked at a constant concentration of 120 μg/mL serving as IS. The standard solutions finally contained 40 mM sodium chloride. The carrier solution (functioning also as an electrophoretic buffer) was freshly prepared. The running buffer was a 15 mM acetic acid–15 mM sodium acetate buffer (pH 5.5) containing 5% (v/v) methanol–3% (v/v) Tween 20. The buffer was prepared daily from stock solution of 0.2 mol/L acetic acid, 0.2 mol/L sodium acetate, methanol and Tween 20, and then adjusted to the desired pH using either 2 M NaOH or 2 M HCl. The final pH of the buffers was checked using a PHS-10A pH meter (Xiaoshan Science Instrumentation Factory, Zhejiang, China). All solutions were filtered through 0.45 μm syringe filters before use.

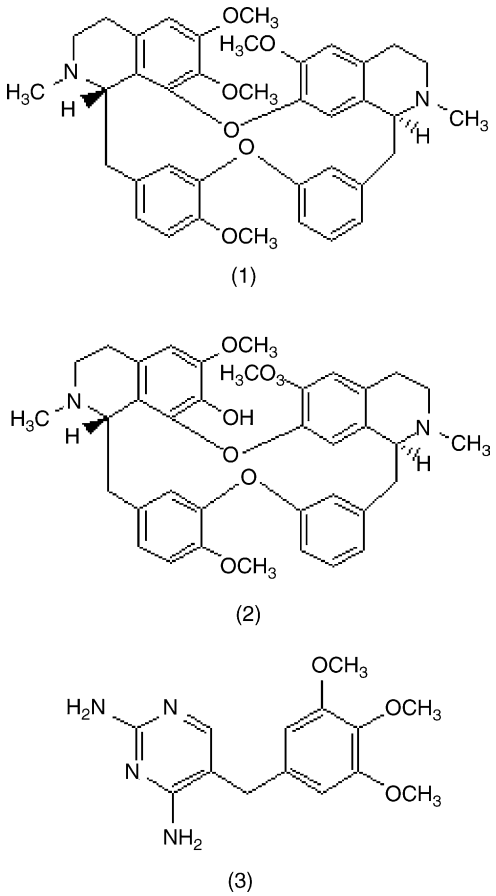

Fig. 1. Structures of (1) TET, (2) FAN and (3) IS.

2.3. Sample preparation

S. tetrandra S. Moore (3 g), Feng Tong An capsule (11 capsules) were extracted by ultrasonic treatment with ethanol (each 25 mL) for 1 h, and filtered through 0.45 μm syringe filters, and then distilled water of equal volume was added to the filtrate, the solutions were diluted with 50% aqueous ethanol to 50 and 30 mL, respectively. The sample solution contained a constant concentration of 120 μg/mL IS. All solutions were filtered through 0.45 μm syringe filters before use.

2.4. Apparatus

A model HPE-100 CE system with 12 kV maximum voltage (Bio-Rad, Hercules, CA) was used for all electrophoretic separations, which was connected to a 486 personal computer with a Chroma chromatography collection system (Bio-Rad) for integration and data treatment. Uncoated fused-silica capillaries of 50 μm I.D., 375 μm O.D. and 29 cm length (25.5 cm effective length) were purchased from Yongnian Optical Fiber Factory (Baoding, Hebei, China). UV detection was carried out at 254 nm.

A K-1000 flow injection analyzer (Hitachi, Japan) was equipped with a peristaltic pump, a 16-way injection valve and a plunger pump. Polytetrafluoroethylene (PTFE) tubing (0.5 mm I.D.) was used for connecting all components of the FI system, including 33 cm length transport line from the valve to the split-flow interface. A sample loop and two reagent loops were made from PTFE. The time period for the injecting sample was defined through man/access mode.

The detailed description of the H-channel microchip has been given elsewhere [19].

2.5. Operation procedure

An unmodified fused-silica capillary was used for all analysis. At the beginning of each working day, the capillary was flushed sequentially with distilled water (5 min), 100 mM NaOH (5 min) and distilled water (5 min), followed by running buffer (5 min) from the capillary outlet reservoir using a water-circulating vacuum pump. Simultaneously, the CE instrument was warmed up until a stable baseline was achieved. Moreover, the capillary was rinsed for 2 min with distilled water, 2 min

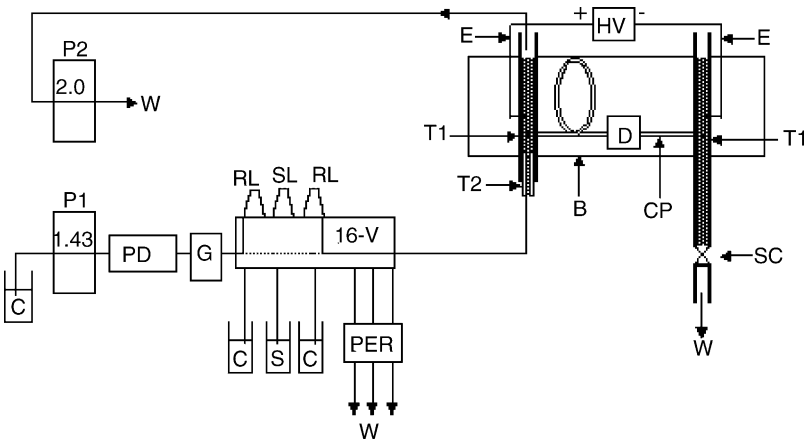

Fig. 2. Manifold for the FI-CE (not to scale). C, carrier solution; S, sample; P1 and P2, pumps; PD, pressure damper; G, pressure gauge; SL, sampling loop; CL, carrier loop; 16-V, 16-way valve; PER, peristaltic pump; B, planar plastic base; T1, Tygon tubing; T2, Tygon tubing (2.0 mL/min); CP, separation capillary column; E, platinum electrode; W, waste; C/S, carrier/sample; HV, high voltage; D, detector; SC, screw clamp.

with 100 mM NaOH, 2 min with distilled water and then 3 min with fresh buffer by manual handling after each run, prior to the next injection. The column was left filled with distilled water overnight.

For the FI operations, the carrier stream was driven by the plunger pump, and in turn was passed through a pressure damper, a pressure gauge and the 16-way valve (as shown in Fig. 2). A sample solution and two carrier solutions (In this study, there was not reaction, and carrier solution was injected into reagent loop.) were delivered to a sample loop and two reagent loops (volume: 20 μL, respectively) in the 16-way injection valve by the peristaltic pump, respectively. Injection of the sample and the carrier solutions into the carrier stream was carried out by changing the flow lines from a dot line to solid lines in the 16-way valve, as shown in Fig. 2. The sample solution was sandwiched by the carrier solutions and transported through the connecting conduit into the T2, where the flow was split and a fraction of the sample zone injected by FI system was introduced into the separation capillary by electrokinetic means for 8 s. A series of samples was injected continuously without interrupting the high voltage (8 kV).

3. Results and discussion

To achieve low limit of detection (LOD) and satisfactory separation, the optimization of separation conditions was of primary importance. In this work, separations of TET, FAN and IS were optimized by studying the effects of the buffer pH, the concentrations of Tween 20 and methanol and applied voltage on the peak areas and resolution between adjacent peaks. The optimum separation of the analytes was obtained with 15 mM acetic acid–15 mM sodium acetate–3% Tween 20–5% methanol buffer at pH 5.5, 20 μL sample volume, 254 nm UV detection, buffer flow rate 1.43 mL/min and 8 kV applied voltage. The peak sequence of the three compounds in MEKC was TET, FAN and IS. The identity of the recorded peaks was confirmed by independent injection of the pure compounds.

3.1. Influence of the buffer pH on separation

To improve the resolution of TET, FAN and IS and decrease the migrate time, we investigated the effect of pH on the resolutions and migrate time of analytes when 15 mM acetic acid–15 mM sodium acetate–2% Tween 20–20% methanol was used as the BGE in the pH range of 3.5–6.0. The results indicated that the resolution between TET and FAN change little, and the resolution between FAN and IS decreased dramatically with the increasing of pH. Also, further increasing the pH of the BGE resulted in rapid elution of the analytes, particularly at pH 5.5. Under normal circumstances, this phenomenon would be attributed to the increase in electroosmotic flow (EOF) with increase in pH [23]. It is well known that the EOF of a native capillary is determined by the degree of dissociation of the silanol groups and the ionic strength of the separation buffer. A higher pH value means a higher degree of dissociation, leading to a higher EOF. Not only resolution of the solutes and migration time but also sensitivity tended to change with the changing of pH. When pH was increased from 3.5 to 5.5, the peak area was increased, and then decreased at higher pH. With concurrent consideration of peak area, resolution and migration time, pH 5.5 was therefore preferred for further studies.

3.2. Influence of Tween 20

Tween 20 is a non-ionic surfactant that has been used to modify the inner wall of the capillary and to suppress the EOF at low pH, and Tween 20 was found, in a previous paper [24], to enhance the solubility of a hydrophobic cationic compound. At pH 5.5, the analytes and IS can still be ionized, and they are present as cations. The critical micelle concentration (CMC) for Tween 20 in pure water is reported to be 0.06 mM [25]; in this study, it can be reasonably estimated that 3% Tween 20 (almost 39 mM) is far exceeding its CMC; so Tween 20 micelles exist predominantly in the BGE solution, leading to the separation mode should be MEKC. To investigate the effect of concentration of Tween 20 on the separation, experiments were performed with concentra-

tion of Tween 20 ranging from 0 to 4% using 15 mM acetic acid–15 mM sodium acetate–20% methanol buffer (pH 5.5), 1.43 mL/min flow rate and 7 kV applied voltage. At 3% Tween 20, the analytes could be separated completely within13.4 min. On considering the resolution and migration time of these analytes, 3% Tween 20 was therefore preferred for further studies.

3.3. Effect of methanol

It was found in the experiments that there was a considerable influence of methanol concentration on the effective mobilities of the analytes and IS. The system without methanol as a modifier, FAN and IS wholly overlapped. When methanol was added in the running buffer, the hydrophobicity and ionic strength of buffer were changed, consequently resulting in the change of the EOF and the resolution. We investigated the effect of methanol contents changed from 0 to 35% on the separation behavior of TET, FAN and IS using 15 mM acetic acid–15 mM sodium acetate–3% Tween 20 (pH 5.5). The effects of methanol contents on migration time and resolution were shown in Fig. 3. As seen in Fig. 3, at 5% methanol, the analytes could be separated completely within12 min. The reason was that the cationic analytes interact with the neutral micelle and the difference in the interaction causes the different migration time of analytes. At 5% methanol, their peaks were narrow and further increasing methanol concentration would make the migration time of the analytes and IS longer and be unfavorable to the analytes' separation in real samples. Thus, 5% methanol was chosen considering the peak shape, resolution and the total analysis time.

3.4. Performance of the combined FI-CE

Preliminary experiments indicated that when the standard solutions were prepared in 50% aqueous ethanol, the repeatability was poor because differences in sample zone and buffer zone conductivity can contribute to electrodispersion and peak asymmetry. To avoid this type of distortion, 40 mM NaCl was added into sample. By this means, the relative standard deviation (RSD) of the determinations was improved.

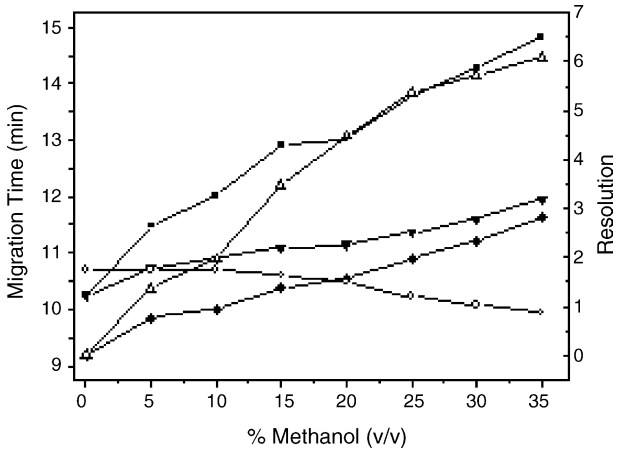

Fig. 3. Influence of methanol concentrations on the resolution of the peaks ((◯) resolution of TET and FAN; (△) resolution of FAN and IS) and migration time ((♦) TET, (▼) FAN, (■) IS). Conditions: 50 μm I.D. × 375 μm O.D. × 29 cm length (25.5 cm effective length), uncoated; buffer, 15 mM acetic acid–15 mM sodium acetate–3% Tween 20 at pH 5.5, voltage, 7 kV; detection wavelength, 254 nm; sample volume, 20 μL; carrier flow rate, 1.43 mL/min; sample: 90 μg/mL TET and FAN; sample solution: 50% aqueous ethanol containing 40 mM NaCl.

The repeatability, linear range and detection limits were studied for the IS method. The RSD for six replicate runs was <3.7% for all of the analytes and the peak area ratios were employed for quantification. The analytes were repeatedly injected into the CE system every 2 min. Six consecutive injections were performed. The electropherograms obtained were shown in Fig. 4. Calibration plots were obtained by plotting the ratio of the peak areas for TET and FAN to IS, versus analyte concentration. Each point on the calibration graph corresponded to the mean value obtained from four independent peak area ratios measurements. The equation of the calibration curve was then used to calculate the concentrations of the analytes in all samples. Six different concentrations of TET and FAN ranging from

Table 1
LOD, RSD of peak area and peak height, regression equation, correlation coefficient, linear range, sample throughput rate for FI-CE system with normal way and IS method ($n = 6$)

	TET		FAN	
	Normal way	IS method	Normal way	IS method
Peak area RSD (%)	3.7	2.3	4.0	1.7
Peak height RSD (%)	2.2	0.4	3.6	2.8
Regression equation[a]				
a	12.904	0.0061	12.291	0.0058
b	−66.126	−0.0341	−54.137	-0.0298
Correlation coefficient	0.9930	0.9996	0.9958	0.9991
LOD (S/N = 3) (μg/mL)	3.3		4.7	
Linear range (μg/mL)	13.0–172.7		13.0–172.7	
Sample throughput rate (h^{-1})	19–40		19–40	

Conditions: 15 mM acetic acid–15 mM sodium acetate–3% Tween 20–5% methanol buffer at pH 5.5, 20 μL sample volume, 254 nm UV detection, buffer flow rate 1.43 mL/min and 8 kV applied voltage.

[a] $y = ax + b$; normal way: y, peak area; IS method: y, the ratio of the peak areas for TET and FAN to IS; x, standard concentration (μg/mL).

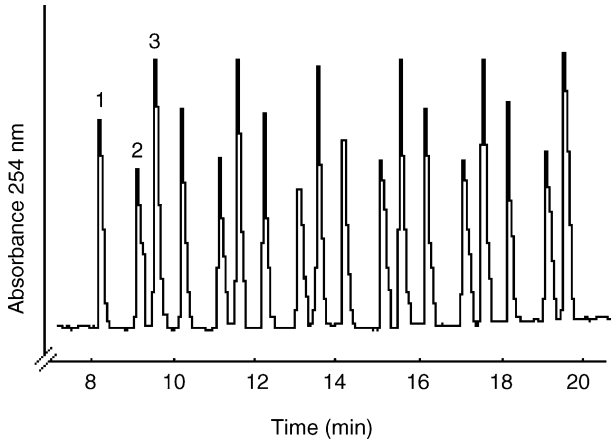

Fig. 4. Recordings of six consecutive injections of a standard mixture of TET, FAN and IS. Peaks: 1 = TET, 2 = FAN, 3 = IS. Dissolution condition: 50% aqueous ethanol containing 40 mM NaCl, sample: 130 μg/mL TET and FAN, 120 μg/mL IS. Separation condition: 15 mM acetic acid–15 mM sodium acetate–3% Tween 20–5% methanol buffer at pH 5.5, voltage, 8 kV; detection wavelength, 254 nm; sample volume, 20 μL; carrier flow rate, 1.43 mL/min.

13.0 to 172.7 μg/mL, and with a constant concentration of IS (120 μg/mL) were prepared by dissolving the standard in 50% aqueous ethanol containing 40 mM NaCl. The performance of this method is summarized in Table 1. As a comparison, the linearity and reproducibility using the normal way were also given. The LOD was calculated as the peak height at a signal-to-noise ratio of 3 (S/N = 3).

3.5. Analysis of herbal samples

To test the applicability of the developed method based on FI-CE system with IS, herbal samples were analyzed. Quantitative analysis was performed under the optimum conditions obtained from the experiments described above. This method was applied to the analysis of TET and FAN in *S. tetrandra* S. Moore and Feng Tong An capsule. The sampling frequency achievable per hour could be estimated according to Fang's equation [26], and, in this work, was calculated as 40 and 19 h^{-1} for *S. tetrandra* S. Moore and Feng Tong An capsule, respectively. The contents of the analytes found in the different kinds of herbs were given in Table 2. The typical electropherograms of the samples were shown in Fig. 5. The peaks were identified by the standard addition methods. The accuracy of the methods and the potential

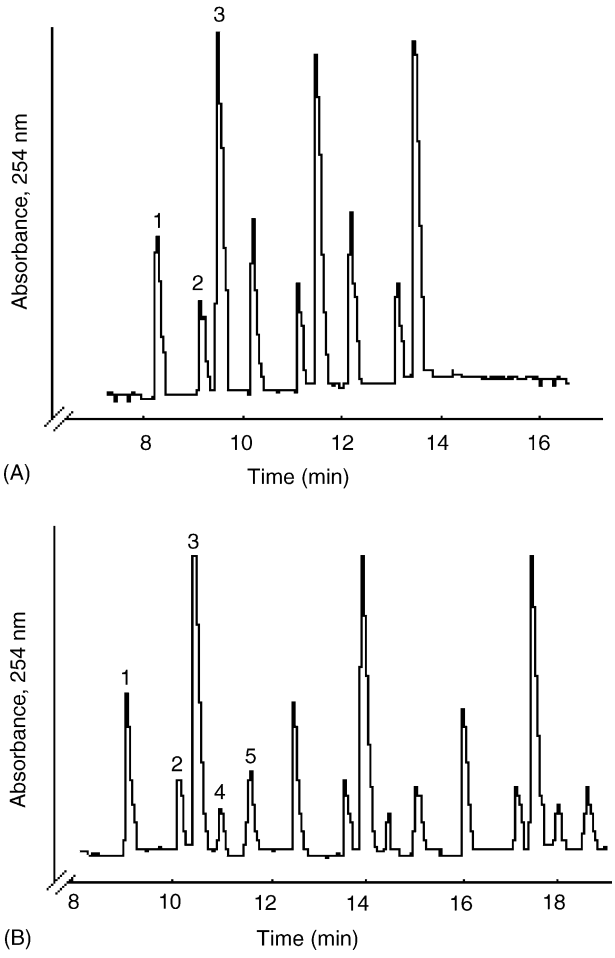

Fig. 5. Electrochromatogram of the herbal medicines. (A) *S. tetrandra* S. Moore; (B) Feng Tong An capsule, Peaks 4 and 5, unknown. Other conditions as in Fig. 4.

Table 2
Results for the determination of the two components in sample extracts (n = 4)

Sample	Ingredient	Content	Concentration spiked (μg/mL)	Concentration found (μg/mL)	Recovery (%)	Average (%)	RSD (%)
Stephania tetrandra S. Moore	TET	1.17 mg/g	10.80	10.76	100	99	4.1
			21.59	22.28	103		
			43.18	40.83	95		
	FAN	0.78 mg/g	10.80	10.62	98	99	3.1
			21.59	20.8	96		
			43.18	43.94	102		
Feng Tong An capsule	TET	0.11 mg/capsule	10.80	11.79	109	101	7.3
			21.59	21.22	98		
			43.18	41.11	95		
	FAN	0.066 mg/capsule	10.80	11.44	106	98	7.5
			21.59	19.95	92		
			43.18	41.23	95		

Conditions: 15 mM acetic acid–15 mM sodium acetate–3% Tween 20–5% methanol buffer at pH 5.5, 20 μL sample volume, 254 nm UV detection, buffer flow rate 1.43 mL/min and 8 kV applied voltage.

matrix effects were established by analyzing spiked samples. The results were presented in Table 2.

4. Conclusions

The result indicates that the proposed FI-CE with IS has also been proven to be a feasible and attractive way for improving sample throughput and providing good accuracy of the quantitation. The separation could be achieved within 12 min (25 min in reference [5]) and real sample throughput rate up to 19–40 h^{-1} in this study. The repeatability was 2.3 and 1.7% with peak area evaluation and 0.13 and 0.11% with migration time evaluation for TET and FAN, respectively. In reference [6], the RSD of the peak area and migration time of each peak were 0.45–4.9% and 0.09–0.59%, respectively. And the correlation coefficient of regression equation with IS was greater than 0.999 for the analytes. The selection of calibration method is critical with electrokinetic injection used for sample introduction in the FI-CE interface.

Acknowledgement

We kindly acknowledge the National Science Foundation of China (No. 20275014) for supporting this work.

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